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(54) Title: PROCESS AND COMPOSITION FOR PERFORMING DNA ASSAYS

(57) Abstract

The present invention provides an improved process for performing nucleic acid hybridization assays that is fully automated. The process utilizes magnetically responsive particles which are derivatized with a ligand.

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PROCESS AND COMPOSITION FOR PERFORMING DNA ASSAYS

BACKGROUND OF THE INVENTION

This invention relates generally to nucleic acid hybridization assays and, more particularly, to a fully automated process for performing nucleic acid hybridization assays using magnetically responsive particles.

Many current diagnostic and epidemiological procedures rely on the culture of clinical specimens, which can be both time consuming and difficult. Immunoassays, such as RIA and ELISA, are faster and are frequently used for viral typing and culture confirmation. Many viruses, however, do not produce significant amounts of antigens and therefore cannot be detected by immunoassays. An alternative procedure for identifying such pathogens employs nucleic acid hybridization assays.

Nucleic acids, which are the carriers of genetic information between generations, are composed of linearly arranged individual units called nucleotides. Each nucleotide has a sugar phosphate group to which is attached one of the pyrimidine or purine bases, adenine (A), thymidine (T), uracil (U) guanine (G) or cytosine (C). Single stranded nucleic acids form a double helix through highly specific bonding between bases on two strands; A will bond only with T or U, G will bond only with C. Thus, a double stranded, or hybridized, nucleic acid will form where, and only where, the sequence of the bases in the two strands is sufficiently complementary as to allow such hybridization. Depending on the form of the sugar phosphate group present, the nucleic acid is termed either deoxyribonucleic acid (DNA) or ribonucleic

acid (RNA).

Nucleic acid hybridization assays are based on this principle of complementarity. Typically, a single stranded nucleotide sequence complementary to the sequence of interest is combined with a sample under conditions allowing hybridization. The former sequence is termed the "probe;" the latter, the "target." The presence of double stranded nucleic acid including the probe indicates that the target sequence is present in the sample. Such assays have wide applicability, including the testing of biological samples for the presence of a pathogen, such as a bacterium, virus or parasite; the diagnosis of disease associated with a genetic abnormality; the indication of susceptibility to certain genetically mediated conditions; paternity or other relatedness, as for example in forensic analysis; and the biological contamination of food or other product.

Where the amount of target nucleic acid in the sample is initially small, procedures may be utilized to amplify the target. Among such amplification procedures is the polymerase chain reaction (PCR) and ligation-based amplification procedures. See, for example, United States Patent Nos. 4,683,195; 4,683,202 and 4,800,159 and the PCT Publication No. WO89/12696.

Various methods are known to those skilled in the art for determining the presence and extent of hybridization. Such assays require that the hybridized probe be distinguishable from non-hybridized probes. The majority of nucleic acid hybridization assays utilize isotopic detection, primarily ^{32}P, and are manual processes requiring separation steps. For example, the conventional membrane-based assays involve sample

pretreatment, denaturation, and fixation of nucleic acids onto solid supports, such as nitrocellulose or nylon filters. Such procedures are imprecise, labor intensive, time consuming, and difficult to automate. Furthermore, the hybridization probes are usually radiolabeled to high specific activity in order to obtain the required sensitivity. Clinical laboratories are averse to such probes because they are unstable and present significant problems of handling and disposal. Consequently, the few such tests on the market have limited practical application in the clinical laboratory.

Non-isotopic labels have also been used in nucleic acid hybridization assays. For example, biotin labeled probes utilizing alkaline phosphatase-avidin polymeric complexes can be used to detect unique DNA sequences immobilized on filters through the enzymatic production of a colored, precipitatable dye, as described by Ward, et al., U.S. Patent No. 4,711,955. These nonisotopic, indirect detection systems however are plagued by intermediate, background-susceptible, binding and washing steps, and are limited to hybridization on membranes.

Several membrane-based nonisotopic direct detection systems have been described utilizing covalently linked enzyme-DNA. Renz and Kurz (Nucl. Acids Res. 12:3435 (1984)) describe long probe methodologies which are limited by lengthy hybridization times and difficult cloning procedures. Alternatively, well defined synthetic oligonucleotide probes can be used. For example, direct labeled enzyme-synthetic oligonucleotide conjugates are described by Jablonski, et al., Nucl. Acids Res. 14:6115 (1986); Kerschner, et al., Am. Soc. Microb., Abst. 56:309 (1987); McLaughlin, et al., Lancet 714, March 28 (1987), which are incorporated

herein by reference. These conjugates are easily synthesized for specific analytes, exhibit unaltered hybridization characteristics with no nonspecific binding and have exceptional sensitivity and stability. Due to higher probe concentrations, oligomer hybridizations are completed more quickly on membrane supports. However, the full utility of these enzyme conjugates has not been realized in conventional membrane formats.

An alternative approach to membrane-based assays is the sandwich assay, as described, for example, United States Patent No. 4,486,539. The target DNA is removed from a crude sample by hybridization to a complementary sequence immobilized on a solid matrix (direct capture) or by hybridization with a ligand labeled probe, followed by capture onto an affinity support (indirect capture) and removal of unbound probe. Bound target is detected by a second directly or indirectly labeled probe specific for a proximal sequence. The addition of this second hybridization step enhances selectivity for the target and reduces the chance of nonspecific interactions. Although the use of long cloned probes limits the utility, this is one of the most appropriate formats for routine diagnostics since it eliminates protracted sample preparation, purification, and nonspecific filter-based target immobilization.

Direct capture hybridization supports are synthesized by attaching nucleic acids, usually cloned DNA, by chemical modification, adsorption, or enzymatic processes to a variety of solid phases, including nitrocellulose, cellulose, nylon, polystyrene, teflon-polyacrylamide, polypropylene, agarose, sephacryl and latex. The hybridization efficiencies and capacities of immobilized DNA have been described in detail. See, for example, Miller, et al., J. Clin. Microb. July 1988, p.

1271-1276 and Yehle, et al., Molecular and Cellular Probes 1:177-193 (1987), which are incorporated herein by reference. The supports described thus far are difficult to make, however, and with the exception of oligonucleotide resins, have relatively poor loading capacities. The mixed-phase direct capture hybridizations are also limited kinetically and may be inefficient due to inaccessibility of immobilized DNA.

In contrast, hybridization in solution is extremely efficient and many times faster than hybridization on solid supports. Solution hybridization with ^{125}I labeled probes, followed by nonspecific adsorption onto hydroxyapatite (HAP) is well known in the art. However, nonspecific background binding to HAP is a function of probe concentration, and capture is nonspecific. Therefore, probe inputs are limiting and hybridization rates are suboptimal.

The DNA assay processes described briefly above are all manual and have not yet proven to be generally effective. Current procedures have suffered from the drawback of not consistently yielding precise and repeatable results. Variations in the relative amounts of the various reagents, the timing of their combination as well as in the needed amount of agitation of the combined reagents, for example, all contribute to the somewhat imprecise and non-repeatable results. In addition, the possibility of contamination and the excessive time and labor required by highly-trained personnel to carry out the numerous and tedious steps of manual assays are further disadvantages inherent in the manual DNA assay process.

It should, therefore, be appreciated that there

is a need for a fully-automated process and reagents for performing DNA assays, which yields highly precise and repeatable results while minimizing the risk of contamination, and which requires minimal intervention and monitoring by highly-trained personnel. The present invention fulfills this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention resides in an improved process for performing nucleic acid hybridization assays that is both highly precise, efficient and repeatable and that is fully automated, requiring only minimal involvement by trained personnel. The process of the invention includes an initial step of providing a programmable XYZ pipetter and an associated plurality of wells, an associated array of thermally-controlled reaction tube holders, each being adapted to carry a test tube, and means for effecting an alternating or rotating magnetic field about each reaction tube's longitudinal axis. The XYZ pipetter is used to transfer nucleic acid samples to separate test tubes carried by the plurality of reaction tube holders, to transfer successively a hybridization solution and a suspension of magnetically responsive particles to the plurality of test tubes at prescribed times, and to effect position of the reaction tubes relative to their associated magnetic fields at prescribed times, such that binding reactions between the target nucleic acid samples, probe and oligonucleotides in the hybridization solution, and magnetically responsive particles can occur and a precise, repeatable automated nucleic acid hybridization assay can be provided.

More particularly, the XYZ pipetter is used by initially placing the hybridization solution in a first well, the suspension of magnetically responsive particles in a second well, a wash buffer solution in a third well, a substrate buffer in a fourth well and a quench buffer solution in a fifth well. The separate nucleic acid target samples are placed in the plurality of reaction tubes, each carried in a separate reaction tube holder. The XYZ pipetter is used initially to transfer the hybridization solution from the first well to the plurality of test tubes, in sequence, whereupon hybridization reactions are allowed to occur. Thereafter, the XYZ pipetter transfers the suspension of magnetically responsive particles from the second well to the plurality of reaction tubes, in sequence, and the reaction tubes are then selectively rotated or the magnetic field removed such that the magnetically responsive particles remain in liquid suspension and are allowed to undergo a binding reaction with the separate nucleic acid samples. Terminating the step of selectively rotating allows the magnetically responsive particles to be moved by the magnetic fields to selected locations in the reaction tubes, allowing the XYZ pipetter then to remove the unbound nucleic acid sample in the liquid phase and hybridization solution from the plurality of reaction tubes. Alternatively, a electromagnetic field may be induced around the stationary tubes to remove the particles from suspension.

The XYZ pipetter then transfers the wash buffer solution from the third well to the reaction tubes. After resuspension and separation of the magnetically responsive particles, the pipetter then removes the wash buffer solution, to leave behind the magnetically responsive particles with bound DNA sample and hybridization molecules. The pipetter then transfers the

substrate buffer from the fourth well to the plurality of reaction tubes whereupon an enzyme probe label catalyzes a detectable reaction. Alternatively, if the label is a luminescent or fluorescent moiety, the probe is dehybridized to allow detection in solution. The pipetter then transfers the quench buffer solution from the fifth well to the plurality of reaction tubes, and then transfers a sample from each of the reaction tubes to a separate well of the microtiter plate. In this fashion, the degree of binding reaction between the hybridization solution and the separate nucleic acid samples can conveniently be measured.

In another, more detailed feature of the invention, the XYZ pipetter is further associated with an optical sensor, and the steps of selectively alternating and fixing the magnetic field are accomplished by moving the pipetter's sampling tip to a location where it can be detected by the optical sensor. By appropriate programming, the precise timing of the steps of selectively alternating and fixing the magnetic field, as well as the time delays between the steps of using the pipetter to transfer the hybridization solution and the solution of magnetically responsive particles, are precisely controlled.

In other, more detailed features of the invention, the hybridization solution includes both nucleic acid probes having an attached label and nucleic acid probes having attached biotin or hapten molecules, and the magnetically responsive particles have attached to them avidin, streptavidin or antibody molecules. The process thereby effects a sandwich assay. The label may be a fluorescent or luminescent moiety, or a labeling enzyme. The labeling enzyme advantageously can exhibit fluorescence or luminescence, and the process can further

include a step of assaying the samples carried by the microtiter plate using a spectrophotometer, fluorometer, or luminometer.

In another aspect, the invention utilizes ligand derivatized magnetically responsive particles to separate single stranded target from heterologous nucleic acid in a hybridization assay. The magnetically responsive particles can have streptavidin stably attached thereto through a covalent spacer. The length and composition of the attachment to the particles is critical to their performance.

Other features and advantages of the present invention should become apparent from the following description of the preferred embodiment process, taken in conjunction with the accompanying drawings, which illustrate, by way of example, the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a perspective view of an XYZ pipetter and an associated array of reagent wells and reaction tube holders, for performing a nucleic acid hybridization assay in accordance with the preferred process of the invention.

FIG. 2 is a plan view of the array of reagent wells and reaction tube holders depicted in FIG. 1.

FIG. 3 is a generalized flowchart depicting the operational steps performed by the XYZ pipetter of FIG. 1, in performing the nucleic acid hybridization assay of the invention.

FIG. 4 is a more detailed flowchart of the operational steps performed by the XYZ pipetter in mixing a hybridization buffer with a number of separate nucleic acid samples.

FIG. 5 is a more detailed flowchart depicting the operational steps performed by the XYZ pipetter in admixing a solution of magnetically responsive particles with the separate nucleic acid samples.

FIG. 6 is a more detailed flowchart depicting the operational steps performed by the XYZ pipetter in washing the reacted nucleic acid samples.

FIG. 7 is a more detailed flowchart depicting the operational steps performed by the XYZ pipetter in transferring the reacted nucleic acid samples to a microtiter plate, for subsequent reading.

DETAILED DESCRIPTION OF THE INVENTION

Reagents appropriate for use in the process of the invention and methods of preparing them are herein described. It will be appreciated that other reagents and processes, known to those skilled in the art, can alternatively be utilized.

Oligonucleotide probes can be synthesized using various standard procedures and reagents. Preferably, the probes are chemically synthesized using methods well known in the art. See, for example, Ruth, PCT Publication No. WO84/03285, which is incorporated herein by reference. Capture probes were prepared containing a biotin moiety attached to either an internal base as described in WO84/03285, or to the 5'-terminal nucleotide through a spacer and a C-6 amino modifier. The amino

terminated oligomers were synthesized using an automated DNA synthesizer, such as Applied Biosystems, Inc. Model 380 (Foster City, CA). Prior to the start of synthesis, spacer phosphoramidite (O-dimethoxytrityl-diethyleneglycol-0'-(cyanoethyl-N,N-diisopropyl-phosphoramidite)) and 5'-amino-modifier C6 [3-(4-monomethoxytrityl- amino)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite], (Glen Research Corp., Herndon, VA) were resuspended in anhydrous acetonitrile.

More specifically, the spacer phosphoramidite was synthesized as follows. Diethyleneglycol (10 g, 94.2 mmole) was rendered anhydrous by repeated evaporation of pyridine under vacuum. 4,4'-dimethoxytritylchloride (3.19 g, 9.4 mmole) in anhydrous pyridine (20 ml) was added with stirring for 1 hour. After vigorous stirring in ice water (100 ml), the reaction mixture was extracted with 75 mls methylene chloride, 3 times. Three back extractions with 75 mls water and drying over sodium sulfate were followed by filtration and rotoevaporation to small volume. The mixture was applied to a SiO_2 column (2.5 X 30 cm), pre-equilibrated in ethylacetate: cyclohexane (1:2 v/v) and eluted in the same solvent. After TLC on SiO_2 (ethylacetate:cyclohexane, 2:1 v/v), the proper fractions were located by UV absorbance and color reaction after HCL vapor exposure. The fractions were combined and rotoevaporated to dryness. The residue was dissolved in 20 mls benzene, extracted 3 times with water and lyophilized (3.16 g, 7.7 mmole O-dimethoxytrityl-diethyleneglycol, 82% yield).

The anhydrous residue was redissolved in tetrahydrofuran (20 ml) and evaporated under vacuum. The residue was redissolved in tetrahydrofuran (25 ml) and diisopropylethylamine (3 ml) and cooled in a dry ice-ethanol bath under argon. 2-cyanoethyl-N,N-

diisopropylchloro- phosphoramidite (2 ml, 9 mmole) was added with stirring (20 minutes) followed by an additional 20 minutes stirring at room temperature. Insoluble salts were vacuum filtered under dry argon and washed with anhydrous tetrahydrofuran. After vacuum concentration, ethylacetate (50 ml) was added to the syrup and extracted 3 times (1 M sodium carbonate, 150 mls each). Drying over sodium sulfate, filtration and concentration were followed with the addition of ethylacetate:cyclohexane (1:2, 5 ml). After elution from a silica column and TLC (as described above), fractions were combined and rotoevaporated to dryness. The residue was lyophilized from benzene and gave an oily residue (2.9 g, 62% yield). The residue was redissolved in benzene, aliquoted and lyophilized in septum capped vials under N_2, and stored at -20°C.

The oligomers were purified by reverse phase HPLC on C-8 silica columns and analyzed by 20% polyacrylamide gel electrophoresis. The oligonucleotide-containing reactive amine spacer arm was reacted for 1 hour with a 100 to 1000 fold molar excess of NHS-X-biotin (N-hydroxysuccinimidyl-aminocaproic-biotin; CalBiochem, La Jolla, CA) in 0.2 M sodium bicarbonate, pH 8.5. Biotinylated probe was purified from free probe and excess biotin by reverse phase HPLC on C-8 silica columns eluted with an acetonitrile gradient, then desalted and ethanol precipitated. Product purity was determined by analytical gel electrophoresis.

Direct labeled alkaline phosphate-DNA conjugates can be prepared according to a procedure adapted from the method of Jablonski, et al. Nucl. Acids Res. 14:6115 (1986), which is incorporated herein by reference. Briefly, the linker arm nucleosides are thymidine analogs modified by replacement of the C-5 methyl with an 11 atom

linker arm which terminates in a primary amine. Such probes are purified by conventional methodologies and exhibit essentially unaltered behavior with respect to physical characteristics as compared to unmodified probes. More specifically, linker arm nucleoside 3'-phosphoramidite was prepare by the method of Ruth, et al. DNA 4:93 (1985), which is incorporated herein by reference, and incorporated into oligonucleotides using an automated DNA synthesizer, such as Applied Biosystems, Inc. Model 380 (Foster City, CA). The oligomers were purified by reverse phase HPLC on C-8 silica columns and analyzed by 20% polyacrylamide gel electrophoresis. Product purity was determined by analytical gel electrophoresis.

Linker arm oligomers were first derivatized with disuccinimidylsuberate (DSS). One volume of oligomer in 0.2 M sodium bicarbonate was combined with a 30 fold excess of DSS in two volumes of DMSO. After 3 minutes at ambient temperature, the reaction was applied to FPLC G-25 gel filtration column and eluted in 1 mM sodium acetate, pH 5.0. The fractions were monitored by flow-through absorptiometry at 260nm, collected and concentrated by microconcentrator (Centricon 10K; Amicon, Danvers, MA).

Sodium chloride crystals were added to the activated probe to a final concentration of 3 M. A two fold molar excess of calf intestine alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) in 3 M NaCl, 0.1 M bicarbonate, pH 8.25 was added and maintained for 16 hours at ambient temperature. The conjugate was purified by FPLC anion exchange and eluted in a NaCl gradient. Peak fractions were collected by 260/280 nm absorption ratios. Analytical nondenaturing polyacrylamide gel electrophoresis confirms product purity.

The invention utilizes ligand derivatized magnetically responsive particles, including those which are magnetic or paramagnetic. A variety of materials can be used, including oxides of iron, chromium and titanium or other metals. The particles should be small enough to remain dispersed, having an average settling time of greater than about three minutes. Preferably, they are about .1 - 10μ in diameter, more preferably about 1μ. Various ligands, other than nucleotides or oligonucleotides can be utilized, including, for example, avidin or streptavidin, biotin, haptens (including, for example, dinitrophenol (DNP) or digoxigenin, with a carrier) lectins, or antibodies (such as those against biotin, fluorescein or digoxigenin).

The length and composition of the covalent linkage between the magnetically responsive particles and the ligands are important to efficient performance. Conventionally, the protein is crosslinked to the solid matrix using nonspecific glutaraldehyde, carbodiimides, or cyanogen bromide crosslinking. Both methods produce the protein coupled to the particle. However, activity is significantly reduced and efficiency and strength of binding in subsequent particle hybridization assays is generally unacceptable. For example, Beebe et al., in PCT Publication #WO 88/02785, use avidin linked to cellulose beads using N,N'-carbonyldiimidazole, and require the use of 33 mg of beads per assay to have adequate binding sites. In PCT Publication #WO 86/07387, Snitman et al., coat anti-fluorescein antibody onto 1/4 inch polystyrene beads by adsorption, and use one bead (equivalent to approximately 120 mg) per assay. In the method described below, the attachment of avidin or streptavidin to magnetically responsive particles produces particles with a binding capacity of approximately 1 X 10^{-9} moles (1 nanomole) of biotin

binding sites per mg of particles. This allows the use of only 15 μg of beads per assay, or less than 1/1000 the amount of particles in the prior art methods. The prior art methods, therefore, cannot provide particles appropriate for use in the invention since it is impractical to use milligrams of beads per assay in terms of cost and performance, and magnetic clearing cannot be accomplished with such large (milligram) amounts of beads.

The covalent linkage produced as described below contains multiple, alternate hydrocarbon and amide (including urea) residues. The linker contains at least three total residues and is preferably less than eight total residues. The method described below produces proteins attached to magnetically responsive particles through a relatively long linkage (20-60 atoms) with a mixed hydrophilic/hydrophobic character. After testing many lengths and compositions of linkage, this linkage has proved to be the most preferred.

Additionally, linkage of proteins to particles using conventional glutaraldehyde, carbodiimide, or cyanogen bromide crosslinking also gives a hydrolyzable linkage which may not be stable to storage conditions in aqueous solutions or mild acids and bases. The present invention provides linkages which are chemically stable to aqueous solutions or mild acids or bases, as, for example between pH 4 and 8 and is additionally stable to many amine-containing buffers, such as Tris, which are often used in biochemical procedures. The resulting particles can be stored and used much more conveniently.

Streptavidin derivatized magnetically responsive particles were prepared. Briefly, 10 ml of aqueous suspension of amine derivatized magnetic particles,

approximately 1 μm in diameter (Advanced Magnetics, Inc., Cambridge, MA) (50 mg/ml) was centrifuged at 12,000 rpm for 30 minutes. The supernatant was discarded and the precipitate was fully resuspended, by vortexing in 20 ml of H_2O and recentrifuged. The particles were further washed in 20 ml portions of water, twice in methanol, 10% methanol/triethylamine, twice in methanol, and finally twice in ether. The particles were completely resuspended each time. The particles were thoroughly desiccated over P_2O_5-NaOH in a vacuum.

Precipitated particles were freely suspended in anhydrous dioxane (10 ml), and then centrifuged off in a corex tube. After removing the supernatant, the particles were resuspended in fresh dioxane (10 ml), then 1,6-diisocyanatohexane (2 ml) was quickly mixed in and the reaction tube placed on a rotator, overnight. Next day, the magnetic particles were centrifuged, the supernatant removed, and the particles were carefully washed with anhydrous dioxane by vortexing and centrifugation, 3 times. Finally, the precipitate was resuspended in 10 ml of dioxane, to which a solution of 1 g 1,6-diamino-hexane in 5 ml of dioxane was added. The reaction tube was placed on the rotator overnight. Next day, the particles were centrifuged and then washed three times with dioxane (20 ml portions). The dioxane-moist particles were resuspended in a solution of glutaric anhydride (1 g), p-dimethylaminopyridine (1 g), anhydrous acetonitrile (15 ml) and anhydrous pyridine (5 ml). The reaction mixture was again placed on a rotator overnight. After centrifugation and removal of the supernatant, the solution-moist particles were resuspended in a mixture of anhydrous pyridine (15 ml) and acetic anhydride (3 ml) and placed on a rotator for 3 hours. The particles were centrifuged and washed twice in pyridine-water (3:1), 20 ml each, then in acetonitrile, 4 times. Finally, they

were resuspended in anhydrous acetonitrile (10 ml) giving a mixture of approximately 50 mg/ml of carboxy-derivatized magnetic particles, which can be stored at -20°C for 1 year or longer.

Three ml of the above suspension were centrifuged. After removing the supernatant, the particles were suspended in dimethylformamide (1.5 ml). A solution of N-hydroxysuccinimide (185 mg), trifluoroacetic anhydride (140 µl) in dichloromethane (2.5 ml) was made, to which 1-methylimidazole (320 µl) was added with cooling and exclusion of moisture. The mixture was diluted with dimethylformamide (2.5 ml) before being added to the magnetic particles prepared above. The mixture was kept in a screw cap vial with teflon linings and placed on a rotator for 6 to 12 hours. The particles were centrifuged and thoroughly washed 6 times with dimethylformamide (10 ml each). They were then transferred into an eppendorf tube and further washed with anhydrous acetonitrile 3 times. They were finally resuspended in anhydrous acetonitrile (1.5 ml). A 100 µl aliquot of suspension was dried down in a desiccator in a vacuum to determine weight of particles per volume suspension.

The activated particles were collected to the side of the test tube by a magnet and the supernatant was decanted. A 20 mg/ml solution of streptavidin in 0.1 M sodium bicarbonate, 0.05% azide was added to the moist particles at 0.24 mgs streptavidin per mg particles. The particles were rotated overnight. The particles were washed four times in 1 X SSC, 0.1% SDS and then brought to 50 mg/ml in storage buffer (1 X SSC, 1 mg/ml BSA, 0.05% triton X100 and 0.05% azide). Protein and 14C-biotin binding assays were performed to determine the streptavidin loading and biotin binding capacity per mg

particles, respectively. Other ligands such as appropriate antibodies, including those to biotin, fluorescein and digoxigenin, can be attached to magnetic particles in a similar manner.

The general procedures of nucleic acid hybridization assays will now be discussed. Preferably, a sandwich type format is used to detect the presence of a particular nucleotide sequence in a sample. Appropriate samples include, for example, cell or tissue extracts, body fluids such as blood or blood products, urine, saliva, food or other material suspected of containing nucleic acids or a particular nucleic acid sequence. Such sample must be treated so that nucleic acids are in solution and available for hybridization. Typically, sample preparation includes lysis of the cells, if present, followed by some separation of cellular nucleic acids from other cellular material. Cells are typically lysed by osmotic pressure, detergent or heat disruption of the membrane, mechanical shearing, ultra sound, or a combination of these methods. Nucleic acids can be purified from other cellular materials using methods of solvent extraction, for example, phenol chloroform, ion exchange, or size exclusion, such as dialysis or gel filtration, centrifugation, or a combination of these methods. For some samples, purification of nucleic acids is unnecessary. As one example, outer cell membranes are lysed by incubation in buffer containing sucrose and non-ionic detergent. Cytoplasmic debris is then removed by pelleting of the nuclei by centrifugation. Addition of detergents and incubation with proteinase K causes lysis of the nuclear membranes and releasing chromosomal nucleic acid. These procedures can be carried out automatically, as described below.

Where small amounts of nucleic acid or the target sequence are present, amplification procedures may be used to specifically increase the target. Amplification is typically achieved using standard procedures such as polymerase chain reaction (PCR), or ligation amplification or other method of amplification. Such methods are well known to those skilled in the art. See, generally PCR TECHNOLOGY, (H. A. Erlich, Ed. Stockton Press, 1989) and United States Patent Nos. 4,683,195; 4,683,202; and 4,800,159, which are incorporated herein by reference.

In PCR primer nucleotide sequences complementary to target sequences on opposite strands flanking the sequence of interest are prepared. In the presence of an appropriate DNA polymerase and nucleotide precursors, and under suitable reaction conditions, multiple copies of the particular target sequences defined by the flanking primers is exponentially generated. Where appropriate a label, such as biotin or enzyme, can be covalently attached to one of the primers, resulting in an amplified labelled oligonucleotide. Alternatively, primer can contain long or short linker arms without interference with PCR procedures.

More specifically, target DNA free of interfering substances is denatured, as by heating to about 95°C, and hybridized with primer oligonucleotides. A polymerase such as taq 1 (Cetus Corp., Emeryville, CA) binds to the hybridized primer sequences and catalyzes the synthesis of new complementary strands in the presence of excess nucleotide triphosphates. The newly formed strands are separated from the template strand by thermal denaturation. As the temperature is lowered, new primers bind to the template and the process is repeated. These repetitive reactions are carried out automatically

in the thermocycler, as described below.

The present invention includes certain novel features which are advantageously exploited in a variety of nucleic acid hybridization formats. The use of magnetically responsive particles to which the target DNA can be bound permits the efficiency of solution hybridization of target and probe while providing a effective subsequent vehicle for the separation of hybridized and non-hybridized nucleic acid. Moreover, the use of magnetically responsive particles facilitates the automation of the assay system. The direct labeling of probes permit ligand/ligand, such as biotin/avidin or hapten/antibody, affinity to be utilized to immobilize the probe/target complex on the magnetically responsive particles.

Preferably, a sandwich type format is used to detect the presence of a particular nucleotide sequence in a sample. For example, biotinylated capture probe, having a sequence complementary to the target sequence or a sequence in proximity thereto is combined with a sample under conditions which permit hybridization between the capture probe and the target. A detection probe comprising a labeled sequence complementary to the target sequence or a sequence in proximity thereto, is also allowed to hybridize with the sample. The affinity of biotin for avidin or hapten to antibody is utilized to attach target/capture probe/detector probe complexes to magnetically responsive particles, where their presence can be detected or quantified.

Alternative sandwich assay formats can also be used, as will be appreciated by those skilled in the art. For example, magnetic or paramagnetic microspheres can be derivatized with other materials, such as biotin, haptens

(including, for example, dinitrophenol (DNP) or digoxigenin with an appropriate carrier, such as BSA), avidin lectins, or antibodies (such as to biotin). The capture probes can, in turn, be derivatized with a non-oligonucleotide moiety complementary to that on the microspheres, such as avidin, antibodies, carbohydrates or haptens. Additionally, various labels can be used on the detection probe, including radioactive, components of an enzymatic reaction, chemiluminescent, bioluminescent and fluorescent moieties.

Preferably the target and the probes are composed of DNA. However, it will be appreciated that the target and/or the probes can be composed of RNA such that the method utilizes DNA-RNA or RNA-RNA hybridization.

With reference now to the drawings, and particularly to FIGS. 1 and 2, there is shown a fully-automated apparatus for performing nucleic acid hybridization assays as described above using magnetically responsive particles and a biotin/avidin conjugate in performing a sandwich assay. The apparatus includes an XYZ pipetter 11, a test tube rack 13, a reaction tube array 15, a plurality of optical sensors 17 associated with the reaction array, a plurality of reagent wells 19, a thermal cycler 21, and a microtiter plate 23. The XYZ pipetter includes a conventional sampling tip 25 for transporting various DNA samples and reagents from one location to another, to perform the assay. Because the apparatus enables the assay to be performed fully automatically, with substantially no intervention required by trained personnel, the assay can be performed to high precision, and with excellent repeatability.

The nucleic acid hybridization performed by the apparatus of FIG. 1 uses a sandwich technology, in which a DNA probe, designed to hybridize with the target DNA, is bound to biotin, while its conjugate, avidin, is bound to magnetically responsive particles. The DNA samples to be tested are first mixed with the biotin-linked DNA probe and a second proximal DNA probe labeled with a reporter group and allowed to react. Thereafter, the mixture is combined with the magnetically-labeled avidin and allowed to react. The magnetic label is then separated from the unreacted DNA sample and DNA probes, and the reporter labeled DNA probe bound to the DNA sample is then detected, to complete the assay.

The XYZ pipetter 11 is a conventional apparatus available from several commercial sources. It is operated under the control of an associated personal computer, which allows great flexibility in selecting the processing details. The pipetter is equipped with a positive-flow washing station 27 for use in cleaning the sampling tip 25 and a liquid level sensor (not shown) on the tip.

As previously mentioned, the apparatus includes a reaction tube array 15 that carries reaction tubes 29 in which the binding reactions are made to occur. In particular, the array includes 48 temperature-controlled reaction tube wells, in a 6 x 8 arrangement, with permanent magnets positioned adjacent to each well. Each well further has associated with it a variable-speed motor capable of alternating clockwise/counterclockwise rotation at 400-to-1000 rpm, through 45-to-1080 degrees of rotation. The reaction tube wells are positioned slightly off center on the vertical motor shaft. Control of each bank of six motors is made using the adjacent

photoelectric switch 17, which can be controlled using the pipetter tip 25. In this fashion, the motors are controlled by mere movement of the pipetter tip, without the need for a separate computer interface.

The additional plurality of reagent wells 19 are provided to accommodate various buffers and reagents, as will be described below. A particle mixer 19a, which includes an associated motor (not shown) for providing vortexing motion, is provided for carrying the magnetically responsive particle reagent. This particle mixer has an associated photoelectric switch 31, for selectively switching the motor on and off, although other types of switches can be used.

The magnetic reaction array 15 holds the various reaction tubes 29 at a selected temperature and functions to selectively disperse and collect the magnetically responsive particles. The particles remain in liquid suspension so long as the tube carrying it is rotated by its associated motor. When the oscillating motion terminates, however, the particles are quickly collected to the sides of the reaction tube under the force of the adjacent permanent magnets, allowing an efficient removal of excess liquid by the pipetter tip 25. The subsequent addition of further liquid reagents and resumption of the oscillating motion causes an immediate uniform resuspension of the magnetic particles.

The thermal cycler 21 is a temperature-programmable microfuge test tube holder adapted to carry out a thermocyclic amplification reaction. Temperature control is preferably provided from about 25° to 110° C, with a temperature ramping speed of about 3 seconds per degree. Amplified samples can be obtained from the thermal cycler for transfer directly into the

hybridization reaction tubes 29 using the pipetter tip 25.

With reference now to the flowchart of FIG. 3, the preferred process for providing a nucleic acid hybridization assay will be described. In an initial step 41, the various nucleic acid samples are manually placed in separate test tubes of the test tube rack 21, a hybridization solution containing a hybridization buffer with oligomers, is placed in a reagent well 19b, and a solution of magnetically responsive particles is placed in the particle mixer well 19a. After this has been done, the XYZ pipetter apparatus is in condition to initiate the fully-automated portion of the nucleic acid hybridization assay.

Thus, in a subsequent step 43, the aspirating/dispensing tip 25 of the XYZ pipetter 11 sequentially aspirates the hybridization solution from the well 19b and the successive nucleic acid samples from the test tubes of the test tube rack 21 and transfers these combined solutions to separate test reaction tubes of the reaction tube array 15. After a prescribed time duration, during which time the hybridization solution is allowed to undergo a binding reaction with the nucleic acid samples, the XYZ pipetter, in a subsequent step 45, aspirates the solution of magnetically responsive particles and transfers it sequentially to the reaction tubes in the reaction tube array.

Thereafter, after a prescribed time duration in which the avidin molecules adhered to the magnetically responsive particles are allowed to undergo binding reactions with the biotin present in each solution sample, the liquid portion of each sample is removed at step 47 and the remaining magnetic particles, with bound

DNA probe and possible DNA sample, is washed using the wash buffer solution carried in a well 19c. In a subsequent step 48, the pipetter tip 25 is used to remove the wash solution from the reaction tubes and to transfer to the tubes a substrate buffer solution. After a prescribed time duration, during which an enzymatic process is allowed to occur, the pipetter tip 25 transfers a quench buffer solution from the well 19d and a sample from each reaction tube to a separate well of the microtiter plate 23 at step 49. The tagged DNA probe in these samples is thereafter read, for example using a fluorometer.

FIG. 4 is a flowchart depicting, in greater detail, the step 43 from FIG. 3 of combining the hybridization solution with the various nucleic acid samples in the reaction tubes of the reaction tube array 13. In particular, in an initial step 51, the pipetter's aspirating/dispensing tip 25 is controllably moved to the well 19b, where it aspirates a prescribed amount of the hybridization solution. Thereafter, in step 53, the tip is moved immediately to the first test tube in the test tube rack 13, where it aspirates a prescribed amount of the nucleic acid sample carried in that test tube. The tip is then moved in step 55 to the first reaction tube in the reaction tube array 15, where it dispenses the combined hybridization solution and nucleic acid sample into the tube. The tip is then washed at the wash station 27, in step 57.

Thereafter, in step 59, it is determined whether or not the last of the nucleic acid samples has been aspirated from the test tube rack 13. If not, and the last tube in the current column has not been processed, the sample number (n) is incremented by one at step 61, and the program returns to the initial step 51 of

aspirating the hybridization solution from the well 19b. Eventually, it will be determined at step 60 that hybridization solution and sample have been dispensed into the last tube in the current column of tubes. When that happens, the program advances to step 63 in which the pipetter tip 25 is moved to the optical sensor 17 for the reaction tube column just completed, to activate its associated column of motors. At step 65 the tip is washed at the wash station 27. The sample number is then incremented and the program returns to step 51 where it aspirates a prescribed amount of hybridization solution. Eventually, it will be determined at step 59 that the last of the n nucleic acid samples has been aspirated from the test tubes of the test tube rack 13 and transferred to a separate one of the reaction tubes of the reaction tube array 15. The program then advances to a step 63, in which the pipetter tip 25 is moved to the optical sensor(s) 17 for the affected column(s) of reaction tubes, to switch on the corresponding motors, to begin agitating the solutions. Finally, at step 65, the tip is washed at the wash station 27.

FIG. 5 is a flowchart depicting, in greater detail, the step 45 in FIG. 3 of adding the magnetically responsive particle solution to the reaction tubes of the reaction tube array 15. In particular, in an initial step 67, the pipetter's aspirating/dispensing tip 25 is moved to the optical sensor 31, to initiate agitation of the particle mixer for the particle mixing well 19a containing the magnetically responsive particle solution. After a prescribed, relatively short time duration, the tip, at step 69, is moved to the optical sensor(s) 17 to inactivate the corresponding column(s) of motors in the reaction tube array 15. The tip then returns to the optical sensor 31, at step 71, to terminate agitation of the particle mixer. At step 73, the tip then aspirates a

prescribed amount of the magnetically responsive particle solution and, at step 75, switches the optical sensor 31 to again agitate the particle mixer. At subsequent step 77, the tip dispenses the magnetically responsive particle solution into the first reaction tube C1, after which the tip is washed at the wash station 27, at step 79.

The program then proceeds to step 81, where it is determined whether or not the magnetically responsive particle solution has been dispensed into the last of the reaction tubes carrying nucleic acid samples. If not, the program proceeds to step 83, where it is determined whether or not the solution has been dispensed into the last reaction tube in the current column of tubes. If not, the reaction tube number is incremented by one, at step 83, and the program makes a repeated pass through the subroutine, for the next reaction tube, beginning with the step 71 of terminating agitation of the particle mixer. Eventually, it will be determined at step 83 that the magnetically responsive particle solution has been dispensed into the last reaction tube in the current column of tubes. When that happens, the program proceeds to step 87, where the pipetter tip 25, switches the optical sensor 17 for the reaction tube column just completed, to activate its associated column of motors. The column number is then incremented at step 87, and the program returns to the step 69 of using the pipetter tip to inactive the next column of motors in the reaction tube array 15.

Eventually, it will be determined at step 81 that the magnetically responsive particle solution has been dispensed into the last of the reaction tubes that contains a nucleic acid sample. When this occurs, the program proceeds to step 91, where the pipetter tip 25

switches the optical sensor 17 for the last reaction tube column, to activate its associated column of motors in the reaction tube array 15. The tip is washed at the wash station 27, at step 93.

FIG. 6 is a flowchart depicting, in greater detail, the washing step 47 of FIG. 3. In particular, in an initial step 95, the pipetter's aspirating/dispensing tip 25 is moved to the first optical sensor 17 to inactivate the first column of motors in the reaction tube array 15. This column is designated by the reference variable X. When the motors stop oscillating the reaction tubes, the magnetically responsive particles are attracted to the sides of the reaction tubes by the adjacent permanent magnets. While this is occurring, the tip 25 is washed at the wash station 27, at step 97. Thereafter, at step 99, the tip is moved to the next optical sensor 17, to inactivate the second, or X + 1, column of motors in the reaction tube array. This allows the magnetically responsive particles to separate from the buffer solution for this second column of reaction tubes while a wash cycle is performed on the first, or X, column of reaction tubes. Next, at step 101, the tip 25 aspirates the buffer solution from the middle portion of the first reaction tube C_1 and, at step 103, discards the aspirated buffer solution and is washed at the wash station 27. At step 105 of the program, it is then determined whether or not the buffer solution has been aspirated from the last of the six reaction tubes of the column X in question. If not, the reaction tube number (n) is incremented by one, at step 107, and the program returns to the step 101 of aspirating the buffer solution.

Eventually, it will be determined at step 105 that the buffer solution has been aspirated from the last

of the six reaction tubes of the column X in question, and the program then will proceed to a step 109, in which the tip 25 aspirates a wash buffer solution from the reagent well 19c, and step 111, where the wash buffer solution is dispensed into all six reaction tubes. The tip is then washed at the wash station 27, at step 113, and the tip then is moved, at step 115, to the first optical sensor 17 to activate the column X of motors in the reaction tube array 15. This begins agitating the six reaction tubes of the column, to homogenize the contained solutions and enhance the washing being effected.

Thereafter, at step 117, it is determined whether or not the variable X, identifying the column number for the six reaction tubes just operated on, is a maximum. If not, the variable X is incremented by one, at step 119, and the program returns to the step 99 of switching the optical sensor 17 for the X + 1 column of reaction tubes, to inactivate the associated column of motors. By the time this step 99 is again reached, the motors for the previous column X of reaction tubes will have been stopped for sufficient time to allow the magnetically responsive particles and buffer solution to separate from each other. The program then proceeds through the subroutine in the same fashion as described above, for this next column of reaction tubes.

Eventually, it will be determined at step 117 that the final column of reaction tubes has been operated on. When that happens, the program proceeds to a step 121, where it is determined whether or not a reference variable k has reached the number four. If not, the variable X is reset to one and the variable k is incremented by one, at step 123, and the program returns to the initial step 99 of switching the optical sensor 17

to inactivate the first column of motors. The program then proceeds through the program subroutine in the same fashion as described above, to effect three wash cycles, until the variable k has reached the numeral 4. When this happens, the step 109 is modified such that the tip aspirates a substrate buffer solution from the well 19d, rather than the wash buffer solution from the well 19c. Eventually, it will be determined at the step 121 that four passes through the program subroutine have been completed, and this program subroutine is then exited.

FIG. 7 is a flowchart depicting, in greater detail, the step 49 of quenching the substrate buffer reaction and the transfer of reaction samples to the microtiter plate 23. In particular, in an initial step 125, the aspirating/dispensing tip 25 of the XYZ pipetter 11 is moved to the first optical sensor 17 to inactivate the first associated column of motors in the reaction tube array 15. This column is designated by the reference variable X. After the motors have stopped oscillating the reaction tubes, the magnetically responsive particles will be attracted to the sides of the reaction tubes by the adjacent permanent magnets. While this is occurring, the tip 25 is washed at the wash station 27, at step 125b. The variable X is incremented by one at step 141. Thereafter, at step 125, the tip is moved to the next optical sensor 17 to inactivate the next column in the reaction tube array. This allows the particles to separate from the buffer for this second column while the samples in the first column are transferred to a microtiter plate. The tip is then washed at the wash station 27, at step 127, and then moved, at step 129, to the reagent well 19e, where it aspirates a prescribed amount of the quench buffer solution. The tip then moves to the first reaction tube of the column of tubes, at step 131, where it aspirates a

sample from the tube, which is then dispensed by the tip, at step 133, into a first well in the microtiter plate 23, at step 117. The program then proceeds to a step 135, where it is determined whether or not a sample has been aspirated from the last of the six reaction tubes in the column. If not, the number n is incremented by one, at step 137, and the program returns to the step 127 of washing the tip 11 at the wash station 27.

Eventually, it will be determined at step 135 that samples have been aspirated from all n reaction tubes in the column of tubes. When this occurs, the program proceeds to step 139, where it is determined whether or not the last column of reaction tubes has been operated upon. If not, the column number X is incremented by one, at step 141, and the program returns to the initial step 125, where the aspirating/dispensing tip 25 switches the optical sensor 17 for the next column, i.e., column X, in the reaction tube assay 15. The program then proceeds through the same subroutine as described above, this time for the next column of reaction tubes.

Eventually, it will be determined at step 139 that samples have been aspirated from all of the columns of reaction tubes in the assay 15. When this occurs, the fully automated portion of the nucleic acid hybridization assay process will have been completed. The microtiter plate 23, which then will carry n reaction samples, is then transported, in step 143, to a suitable fluorimeter, to provide an accurate and precise measure of the degree of reaction for each DNA sample.

The sequential operation of the XYZ pipetter 11, as described above with reference to the flowcharts of FIGS. 3-7, can be accomplished using a computer program

appropriately written to interface with the particular XYZ pipetter being used. An accompanying appendix is a printout of the source code for one suitable program written to operate on an IBM PS-2 Model 30-286 personal computer, or equivalent. The program is written in C, for use with a conventional Packard XYZ pipetter.

Additional operations can also be performed by appropriate programming of the XYZ pipetter. As indicated an amplification procedure can be performed prior to placing the samples in the test tubes. Similarly, samples requiring preparation prior to hybridization, as for example cell lysis, can be placed directly in the reaction tubes and appropriate solutions added and removed therefrom.

The following examples are intended to illustrate, but not the invention.

EXAMPLE I

Detection of Cloned HIV Fragment

A model system was constructed in which a human immunodeficiency virus (HIV) fragment was inserted into a cloning vector. Briefly, a SP6-HIV vector (pBH10-R3) was obtained from E.I. Du Pont & De Nemours, Wilmington, DE. The vector was constructed by cloning a 9 Kb of HIV genomic insert into the Sst-1 restriction site of PSP-64. The plasmid pMBI107 was constructed by restriction of pBH10-R3 with Bam-H1 and then religation which resulted in the deletion of the 3'-LTR region. Cells were transformed and selected for ampicillan resistance. Plasmid was purified by CsCl gradient centrifugation (Maniatis, et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, New York (1989), which is incorporated herein by reference).

pMBI107 was linearized by restriction with Bam-H1. A dilution series of Bam H1-pMBI107 was made in 10 mM Tris, pH 7.5, 1 mM EDTA (TE). Ten μl of each target dilution was added to 10 μl of 0.01 mg/ml human placenta (HP) DNA in TE. Controls contained equal volumes of TE and HP DNA. Samples were denatured at 95°C for 10 minutes, chilled in an ice water bath for 1 minute and centrifuged briefly to collect condensate. The samples were placed in tubes in the magnetic sample rack on the worktable.

Biotinylated and acid phosphatase labelled oligonucleotides were prepared as described above, using sequences listed in Table I. A processing program for the XYZ pipetter was iniated and prompted for assay variables, such as a sample number, replicate number, etc. The XYZ pipetter automatically combined 102 μl of hybridization buffer (6XSSC, pH 8.0, 10% formamide, 0.1% SDS, 0.1 mg/ml BSA) 3 nM biotin-N231, -N226, -N224, -N211, -N253 and -N229, and 3 nM AP-N218, -N220, -N227, -N233 and -N234) and 20 μl of sample into reaction tubes in the magnetic rack. The sampling tip washed itself between each sample to eliminate the possibility of carryover. Hybridizations proceed at 37°C with rotation for 30 minutes.

The XYZ pipetter then automatically added 15 μl of a 1 mg/ml streptavidin-particles (SA-particles), prepared as described above, in 6XSSC, pH 8.0, 10% formamide, 0.1% SDS, 0.1 mg/ml BSA to each reaction tube. Fifteen μg of SA-particles posses a biotin binding capacity of approximately 60 pmoles. Thus, there is a 35 fold molar excess of biotin binding sites over biotin probe. Capture of the ternary hybrid complex onto the particles proceeds at 37°C with rotation for 30 minutes.

TABLE I

Probe Sequences

N178	5'-dATA ATC CAC CTA TCC CAG TAG GAG AAA T
N279	5'-dTTT GGT CCT TGT CTT ATG TCC AGA ATG C
N280	5'-dCAT TCT TAC TAT TTT ATT TAA TCC CA
N231	5'-dCTA GGT GAT ATG GCC TGA TGT
N226	5'-dCCC TAT CAT TTT TGG TTT CCA T
N224	5'-dTGT TGA CAG GTG TAG GTC CTA
N211	5'-dCTG GCT TTA ATT TTA CTG GTA CA
N253	5'-dTGC CAT TTG TAC TGC TGT CTT
N229	5'-dTGC CAC ACA ATC ATC ACC TGC
N218	5'-dTAG AGG GTT GCT ACT GTA TTA T
N220	5'-dTAG TTC CTG CTA TGT CAC TTC C
N227	5'-dCTG TCT BTAC TTT GAT AAA ACC TC
N233	5'-dTAT TCT TTC CCC TGC ACT GTA
N234	5'-dCTG TAA TAA ACC CGA AAA TTT TGA
A105	5'-dCCC GAG CCG ATG ACT TAC TGG C
A214	5'-dGAT ATC TCA CCC TGG TCG AGG CGG T
A209	5'-dTGT GTG GTG TAG ATG TTC GCG ATT G
N174	5'-dCAG GAG CAG ATG ATA CAG TAT TAG
C103	5'-dAGG CGT TTC CAC ATC TAT ATA GT
C204	5'-dAGT ATC ATC ACC CAC GAT GTG CT

The XYZ pipetter automatically stopped the rotation of the reaction tubes by tripping a photoswitch with the sampling tip. Upon cessation of rotation the particles were quickly cleared from solution by the integral side mounted permanent magnets. The XYZ pipetter automatically aspirated the buffer from each reaction tube, washing the tip between each sample, in a given row. The XYZ pipetter automatically aspirated and dispensed 200 μl wash buffer (4 X SSC, 0.1% SDS) into each reaction tube, washing the tip between each sample. The XYZ pipetter then tripped the photoswitch to commence rotation of the tubes in a given row. The XYZ pipetter

proceeded to wash the next row of tubes as described above until all the samples have been processed. This process was repeated 2 more times for a total of 3 washes, each at 37°C for 5 minutes.

The above wash process was repeated once more with the variation that 150 μl of substrate buffer (30 μM 4-methylumbelliferyl phosphate (4-MUBP), JBL Scientific, San Louis Obispo, CA) in 0.1 M diethanolamine, (DEA), pH 9.0 (JBL), 5 mM $MgCl_2$,) was dispensed into each reaction tube rather than wash buffer. Incubation with substrate proceeded at 37°C with rotation for 60 minutes.

Again, the sampling tip pipetter automatically tripped a photoswitch to stop the rotation of the reaction tubes in a given row, allowing the particles to clear from solution. The XYZ pipetter aspirated 40 μl of 100 mM EDTA from a reagent rack and 110 μl of substrate buffer from the reaction tubes and dispensed the liquid into a well in a microtiter plate. The XYZ pipetter performed a tip wash between each sample. The XYZ pipetter proceeded to quench/transfer the next row of tubes until all the samples were processed.

At the end of the program, an alarm sounded to alert the user that the assay was completed. The microtiter plate was used on a fluorescent plate reader (Pandex FCA, Baxter Healthcare, Pandex Division, Mundelein, IL) which automatically scanned each well. Alternatively, a single well fluorometer can be used manually. The plate can be read immediately or up to 16 hours after completion. The results are presented in Table II.

The results indicate that the minimum extrapolated detection limit based on a signal to noise

ratio of 2.0 is 4 X 10^5 copies pMBI107 plasmid target. The results are linear with respect to target level over three orders of magnitude. Multiple capture/reporter probe pairs increase signal without a proportional increase in noises resulting in better sensitivity overall.

TABLE II

pMBI107 Dilution Series: Multiple Sandwich Pairs

Copies target	Relative Fluorescent Units	Signal/ Noise
0	4	
500,000	10	2.5
5,000,000	66	16.5
50,000,000	522	130.5
500,000,000	4517	1129.3

EXAMPLE II

Automated Amplification and Detection of Viral DNA

In this example of the invention, it was demonstrated that it was possible to combine a semi-automated target amplification assay with the automated sandwich assay in a HSV model system. It was demonstrated that open tube amplification could be performed by the XYZ pipetter without significant contamination or carryover.

Due to the sensitivity of target amplificaton, it is extremely susceptible to inter- and intra-assay contamination. Amplification reactions were set up in a

clean (i.e. no exposure to amplification products) biosafety hood in a room separate from the XYZ pipetter. Clean positive displacement pipettes (PDP) with disposable pistons and capillaries (Rainin, Woburn, MA), sterile tissue grade water (Sigma Chemical Co., St. Louis, MO), sterile light mineral oil (Sigma) and sterile 0.6 ml snap cap tubes (Robbins Scientific, Sunnyvale, CA) were used to minimize contamination.

Fresh Taq 1 heat stable, recombinant DNA polymerase (3.1 units AmpliTaq Cetus Corp., Emeryville, CA) was added to 80 μl amplification buffer (62.5 mM KCl, 12.5 mM Tris-HCl, pH 8.3, 1.88 mM $MgCl_2$, 0.13% (w/v) gelatin, 250 μM dNTP, 0.31 μM biotin-A105 and 1.25 μM A214). Twenty μl water and 20 μl template (1 X 104 copies pHSV106 (Bethesda Research Laboratories, Gaithersburg, MD)) was added to negative and positive samples, respectively. The final reaction mix contained 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM MgCl2, 0.1% (w/v) gelatin, 200 μM each dNTP (Pharmacia, Piscataway, NJ), 0.25 μM biotin-A105 forward primer and 1.0 μM A214 reverse primer and 2.5 units AmpliTaq). Samples were overlaid with 100 μl light mineral oil. Amplification control tubes were capped whereas experimental tubes were left uncapped and placed on a thermal cycler (Ericomp, San Diego, CA) which had been modified to fit on the work table of the XYZ pipetter. An amplification and sandwich protocol were run concurrently, the sandwich containing a 210 minute timer delay. The amplification protocol required approximately 200 minutes to complete and consisted of the following steps: 1 minute denaturation at 94°C; 30 amplification cycles of 1 minute at 55°C and then 1 minute at 94°C; 5 minute extension at 72°C; 10 minute denaturation at 95°C. The process yielded 1 X 10^9 fold amplification of target at 100% efficiency.

Amplification products were analyzed by agarose gel electrophoresis, dot blot, Southern blot and sandwich, the latter as described in Example I, using as the detector probe AP-A209. The latter was the only method that produced quantitative results.

At the end of the amplification program, the closed tubes were manually uncapped. The XYZ pipetter automatically inverted the aqueous and oil phases by dispensing 200 µl chloroform, equilbrated in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. The oil/chloroform layer sank to the bottom and the aqueous layer floated to the top. The XYZ pipetter then combined 102 µl hybridization buffer (6 X SSC, pH 8.0, 10% FAM, 0.1% SDS, 0.1 mg/ml BSA) containing 3 nM AP-A209 and 20 µl of amplification products from the thermal cycler. The automated sandwich assay proceeded as described in Example I. It is important that the total amount of biotin (free biotin primer and biotinylated amplification product) in the sandwich assay does not exceed the binding capacity of the SA-particles. Twenty µl of a 0.25 µM biotin primer solution is equivalent to 5 pmoles of total biotin which is 12 times less than the 60 pmoles binding capacity of the particles.

The results indicated that it was possible to perform target amplification on the worktable of a XYZ pipetter followed by an automated sandwich assay. The phase inversion process allowed the sampling tip to aspirate the sample without contacting the oil layer which markedly reduced the carryover frequency. The contamination frequency (cf) was defined as the number of open negative samples that gave relative fluorescent units (rfu) greater than twice the rfu from closed negative samples.

$$C = \frac{\text{\# open negative rfu} > 2 \text{ X closed negative rfu}}{\text{total \# open negative}}$$

CF = 0/47 (Average results from nine (9) separate experiments)

Data from a representative open tube amplification assay is shown below.

Tube Status	+ or - Target	Average Relative Fluorescent Units (actual)
closed	-	20 (22; 18)
	+	22,742 (22,900; 22,584)
	-	43 (44; 43) (average closed neg. = 32 rfu)
open	-	42 (26; 58)
	+	23178 (22,610; 23,746)
	-	50 (56; 44)
	+	22991 (22,952; 23,030)
	-	54 (54; 54)
	+	22,694 (22,894; 22,694)
	-	36 (32; 40)

$$cf = \frac{\text{\# open negative rfu}}{4} > 64 \text{ rfu} = 0/4$$

EXAMPLE III

Detection of HIV in Cultured Cells

HIV infected CEM-CM3 cells (ATCC Accession No. TIB 195) containing approximately 1 to 20 copies of HIV virus/cell were harvested and prepared for PCR as

described below. Alternatively, reverse transcriptase could be used to form cDNA from RNA which could then be used in PCR.

CEM-CM3 cells were cultured in 75 cc flasks in RMPI 1640 culture media (M.A. Bioproducts, Walkersville, MD), 10% fetal calf serum, 1% penicillin-streptomycin, 1% Fungizone (M.A. Bioproducts) and 1% L-glutamine at 37°C in 5% CO_2. Cells were passed 1:6 every 5 to 6 days. Control cells were passed as follows: 5 mls CEM-CM3:30 mls RMPI media. HIV infected CEM-CM3 cells were passed as follows: 5 mls HIV infected CEM-CM3 cells: 10 mls CEM-CM3 and 20 mls RMPI media. Uninfected CEM-CM3 cells were added to infected cells to increase th viral titer. To maintain a HIV CEM-CM3 continuous cell line without increasing the viral titer, add 5 mls HIV infected CEM-CM3 cells to 30 mls RMPI media. Cells were harvested after 4 to 5 days when titer was maximum. Cells were pelleted in a clinical centrifuge at 1000 rpm for 15 minutes. The supernatant was discarded and the pellet was resuspended in 5 mls 1 X PBS. Cell concentration was quantitated by hemacytometry, usually 2-5 X 10^6 cells/ml. Concentration was adjusted to suit.

A dilution series from 1 X 10^1 to 1 X 10^5 HIV infected CEM-CM3 cells were spiked into 1 X 10^4 uninfected CEM-CM3 cells. The cells (Vf = 65 μl) were lysed as follows: 500 μl outer membrane lysis buffer (10 mM Tris-Cl, pH 8.0, 1.5 mM $MgCl_2$, 140 mM NaCl and 0.5% NP-40) was added and the mixture was vortexed and centrifuged in a microfuge. The pellet was resuspended in 50 μl nuclear membrane lysis buffer (50 mM KCl, 10 mM Tris-Cl, pH 8.3, 2.5 mM $MgCl_2$, 0.1% (w/v) gelatin, 0.05 mg/ml proteinase K, 20 mM DDT and 1.7 μM SDS) and incubated at 55°C for one hour. The samples were boiled for 20 minutes to inactivate the proteinase K and centrifuged to collect

the condensate. In a laminar flow hood, 25 μl of the sample mix was added to 75 μl of amplification buffer (67 mM KCl, 13 mM Tris-Cl, pH 8.3, 2.0 mM $MgCl_2$, 0.13% gelatin, 267 μM each dNTP, 0.33 μM biotin-N174, 1.3 μM N224 and 3.3 units freshly added AmpliTaq; Cetus Corp., Emmeryville, CA) for a final concentration of 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 0.1% (w/v) gelatin, 200 μM each dNTP, 0.25 μM biotin-N174 forward primer and 1.0 μM N224 reverse primer and 2.5 units freshly added heat stable recombinant DNA polymerase (AmpliTaq; Cetus Corp., Emeryville, CA). The sample tubes were placed on a thermal cycler and amplified as follows: 1 minute denaturation at 94°C; 30 amplification cycles of 1 minute at 55°C and then 1 minute at 94°C; 5 minute extension at 72°C. A 20 μl aliquot of the amplified product was assayed in sandwich using detector probe AP-N227 as described in Example I and II.

Experimental results indicate that 10 HIV infected cells could be detected, albeit inconsistently, by automated sandwich assay after target amplification. The amount of signal generated is not proportional to the amount of target present. This may be due to limiting reaction conditions and/or inhibition by cellular components during amplification. This latter is supported by the fact that 1 X 10^5 infected cells resulted in complete inhibition. The sandwich assay was not limiting because there was a 10 fold molar excess of biotin binding sites on the particles and because complete turnover of MUBP results in about 70,000 rfu.

TABLE III

HIV-CEM per 10,000 CEM	Relative Fluorescent Units (rfu)	Average Fluorescent Units	Standard Error
10	1700		
	28	584	967
	24		
100	12352		
	19586	17111	4123
	19396		
1,000	22060		
	21592	21825	234
	21824		
10,000	23470		
	17494	22041	4028
	25160		
100,000	8		
	14	17	11
	30		

EXAMPLE IV

Detection of HIV in Seropositive Blood Samples

In this example of the invention, it was demonstrated that the automated system was able to detect HIV virus in blood samples from seropositive patients.

Blood was collected from the donor by withdrawal into a 10.0 ml heparinized or EDTA-treated vacutainer. The samples were stored at room temperature up to 24 hours after withdrawal.

The blood vacutainer was inverted several times to mix well. Approximately 7.0 ml whole blood was removed by volumetric pipette. The blood was transferred to a cell separation tube (LeukoPrep Cat. No. 2750-2752, manufactured by Becton-Dickinson, Rutherford, NJ) and

capped with rubber stopper provided by manufacturer. An additional 2.0 ml of 1 X PBS (120mM NaCl, 2.7mM KCl, 10mM phosphate buffered salts) was added to bring the total volume to 9.0 ml. The LeukoPrep tube was centrifuged at 1600 X g for 20 minutes at room temperature in rotor and centrifuge designed for containment of infectious material. After centrifugation, the upper layer of plasma was removed and discarded. The "buffy coat" containing mononuclear cells was removed and transferred to a 1.5 ml eppendorf tube. From this, 0.5 ml mononuclear cells was transferred to a 1.5 ml screw-cap eppendorf tube. The remaining cells were discarded. Alternate methods of mononuclear cell isolation may be used, such as Sepracell density gradient method and Ficoll-Hypague.

One half ml of Lysis Buffer (50mM KCl, 10mM Tris-Cl, pH 8.3 (25°C), 2.5mM $MgCl_2$, 0.1mg/ml gelatin, 0.45% NP40, 0.45% Tween-20, and 60 μg/ml proteinase K added just before use) was added to the nuclear pellets. The nuclei were resuspended by vortexing and/or disruption by pipette mixing. Optimal proteinase K activity was promoted by incubation at 55°C for one hour. Proteinase K was inactivated and genomic DNA fully denatured by boiling the samples for 20 minutes. The sample was collected by brief centrifugation.

Amplification of target DNA was performed as follows: Seventy-five microliters of amplification buffer as described in Example III was added to a 0.6ml eppendorf tube (Robbins Scientific). Twenty-five microliters of cell lysate (approximately 10^5 cells or lug genomic DNA) was added and pipette mixed. The final reaction mix contained 10mM Tris-Cl, pH 8.3 (25°C), 50mM KCl, 1.5mM $MgCl_2$, 0.1% (w/v) gelatin, 200 μM each dNTP (Pharmacia), 0.25 μM biotin-N178 forward primer, 1 μM

N279 reverse primer and 2.5 units AmpliTaq (Cetus). The sample was overlayed with 100ul oil (Sigma light). The samples were amplified in an Ericomp Programmable Cyclic Reactor (San Diego, CA) as described in Example III.

Amplified product for assay in the automated sandwich hybridization system was prepared as follows: Twenty-five microliters of HIV amplified product was added to 25 μl 10 mM Tris, pH 8.0 in a 2.0 ml microfuge tube. This dilution provides an analysis of 1/10 of the PCR reaction. Sandwich proceded as described in Example I using AP-N280 as the detector probe. The results are presented in Table IV.

TABLE IV

Patient ID	Relative Fluorescent Units
106-009	41800
016-167	28422
081-046	24720
106-026	45506
neg 1	2986
	2970
neg 2	2620
	2582

The above data represents a single experiment. The cutoff for a positive result was made at 2X the highest negative control for that assay. Even though the negative controls were unusually high, all 4 results correlated with serology.

EXAMPLE V

Detection of Campylobacter

In this example of the invention, it was demonstrated that the automated sandwich assay could detect 5s RNA purified form campylobacter infected stool samples.

Several campylobacter infected stool samples were analyzed using conventional dot-blot analysis. Briefly, 75 µl denaturation reagent (0.2 N HCl) was added to the 2 ml eluates from an ion exchange column (Extractor-10™, Molecular Biosystems, Inc., San Diego, CA) and was then denatured for 5 minutes at room temperature. The denatured samples were applied to an equilibrated centrifuge tube having a transverse membrane (Gene Screen Plus; DuPont/NEN, Boston, MA). The device was spun at 750 X g for 20 minutes. The membrane was removed and placed in fixing reagent (1 M sodium carbonate) for 30 seconds. It was stirred in distilled water for 30 seconds and blot dried on Whatman 3 MM paper. The membrane was transferred to a hybridization bag and pre-hybridized for 15 minutes at 50°C in 2 mls hybridization buffer (0.75 M sodium citrate, 1% sarcosine, 0.5% BSA, 0.6% Kathon (Rohm and Haas, Philadelphia, PA)). The buffer was discarded and 2 mls fresh Hybridization Buffer were added, containing 2.5 nM AP-C103b. Hybridization was allowed to proceed for 15 minutes at 50°C. The product was washed for 20 minutes at 50°C in prewarmed working membrane wash buffer 1 (2 X SSC, pH 7.0, 0.5% sarcosine, 0.12% Kathon) and washed 10 minutes at room temperature in working membrane buffer 2 (1 X SSC, pH 7.0, 0.5% triton X100 0.12% Kathon). The membrane was placed in a plastic bag and developed colorimetrically with freshly added (Nitro Blue Tetrazolium; (NBT; Ameresco, Solon, OH) and 5-Bromo-4-

Chloro-3-indolyl-phosphate, (BCIP; Ameresco) in substrate buffer (3.7 μM NBT, 4.6 μM BCIP, 0.1 M tris-Cl, pH 7.5, 0.1 M NaCl, 0.05 M $MgCl_2$, 0.02% NaN_3) for 4 hours at 37°C. A weak and a strong positive and a negative control were identified.

A conventional manual column and a semi-automated bulk affinity chromatography purification method were compared by an automated magnetic sandwich assay. For the manual column format, 200 μl of sample, 800 μl diluent reagent (10% formalin, 0.1 M sodium phosphate, pH 7.0) and 2000 μl lysis reagent (8 M urea, 0.25% SDS, 0.25% sarcosine, 0.05 M EDTA) were combined and incubated 30 minutes at 50°C. The sample was applied to a pre-equilibrated extractor column containing 1 ml of anion exchange resin. The column was washed with 15 mls Wash Reagent 1 (40% ethanol, 0.2 M NaCl, 0.02 M Tris-Cl, pH 7.5), 5 mls Wash Reagent 2 (0.25 M NaCl, 0.02 M tris-Cl, pH 7.5, 0.05% NaN_3) and eluted in 2 mls Elution Reagent (0.5 M NaCl, 0.02 M Tris-Cl, pH 7.5, 0.05% NaN_3). The eluent was concentrated to 330 μl by lyophilization. The sample was adjusted to the following final concentrations: 10% formamide, 0.1% SDS, 3.75 nM biotin-C204b in a final volume of 400 μl. The sample was denatured 10 minutes at 95°C, chilled in ice water, spun briefly to collect condensate and placed in the XYZ pipetter test tube rack.

For the semi-automated bulk format, two hundred μl of sample, 100 μl Diluent Reagent and 600 μl Lysis Reagent were combined and incubated 30 minutes at 50°C. A portion of the sample (275 μl) was applied to 300 μl of equilibrated 50% matrix. The sample tubes were placed in the magnetic rack and agitated 10 minutes at 37°C. The tubes were automatically turned off and the matrix was allowed to settle for 5 minutes. The sampling tip

aspirated 300 µl which left some liquid over the matrix bed. The sampling tip added 300 µl Wash Reagent 1, the tubes were agitated for 5 minutes then turned off, and the matrix was allowed to settle and 300 µl was aspirated. The procedure was repeated again with Wash Reagent 1 and repeated twice with Wash Reagent 2. Target was eluted from matrix with 400 µl 8.33 X SSC. 330 µl of sample were aspirated. The sample was adjusted to the following final concentrations: 10% formamide, 0.1% SDS, 3.75 nM biotin-C204b in a final volume of 400 µl. The sample was denatured 10 minutes at 95°C, chilled in ice water, spun briefly to collect condensate and placed in the sample rack.

The XYZ pipetter program was loaded and prompted variables were keyed in by the operator. The sampling tip automatically combined 100 µl hybridization buffer, containing 15 nM AP-C103b, and 400 µl into reaction tubes. The remainder of the assay proceeds as described in Example I with the variation that 2 X SSC was substituted for 1 X SSC in the wash buffer.

Results indicate that a semi-automated sample preparation and sandwich hybridization method was feasible using the magnetic rack and a XYZ pipetter. The bulk chromatography format compares favorably with the column format. Sample #1270c was more viscous than the others and this may have contributed to decreased signal in the bulk format.

TABLE V

Campylobacter Sandwich-Column vs.
Bulk Sample Purification

Sample Type	Sample I.D.	Relative Fluorescent Units (Actual) Column	Bulk	Bulk/ Column
negative	1780	52(36; 68)	35 (34; 36)	67%
weak positive	1791	176(184; 168)	293(252; 334)	167%
strong positive	1270c	5319(5424; 5214)	3140(3072; 3208)	59%

EXAMPLE VI

Automated Sample Preparation by
Affinity Capture of HIV Target

In this example of the invention, the sample preparation and concentration was achieved using the automated system and an affinity capture method. The advantages of the method include target enrichment, which can obviate the need for amplification, and compatibility with automation. The example described below detects HIV DNA in blood.

Blood samples were collected, transferred to Leucoprep tubes, and centrifuged as described in Example IV. The caps were removed and the tubes were placed in a sample rack on the worktable of the XYZ pipetter, which was properly programmed to perform the following steps. The sampling tip transfered 1 ml of mononuclear cells from the "buffy coat" to a reaction tube in the magnetic rack. The cells were lysed and the nucleic acid denatured by adding 1 ml 4.0 M guanidinium thiocyanate, 10 mM EDTA, pH 8.0 and incubated for 10 minutes at 37°C

with agitation. Biotin capture probe in hybridization buffer (15 X SSC, 25% formamide, 0.125% SDS, 0.125% sarcosin, 0.25 mg/ml BSA and 7.5 nM biotin-N178) is added and the tubes were agitated for 30 minutes at 37°C. Thirty μg paramagnetic streptavidin derivatized Fe_3O_4 particles (Advanced Magnetics) were added and agitated for 30 minutes at 37°C. Upon cessation of agitation, the particles containing bound biotin capture probe hybridized to target nucleic acid were collected to the sides of the tubes. The supernatant containing heterologous nucleic acids and cellular debris was aspirated. The particles were washed twice for 5 minutes each in 200 μl of 20 mM Tris, pH 8.3, 250 mM KCl with agitation. The target nucleic acid was released from the particles by denaturing the capture probe:target hybrid with 20 μl 0.25 M KOH, followed by neutralization with 20 μl 0.25 M HCL, 150 mM Tris, pH 8.3 for 5 minutes each with agitation. The addition of base and acid was reversed if RNA was the target of interest. The supernatant (40 μl) was transferred to a fresh tube for subsequent use with amplification followed by sandwich assay, as described in Example IV.

This procedure effectively concentrated the amount of target approximately 200 fold in an automated format. The method is compatible with many types of samples and sample preparation treatments.

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

WE CLAIM:

1. In a process for performing a nucleic acid hybridization assay, the improvement comprising:

providing a programmable XYZ pipetter having an aspirating/dispensing tip, and further providing an associated plurality of wells and an associated array of test tube holders, each test tube holder being adapted to carry and selectively rotate a test tube about its longitudinal axis, within a magnetic field; and

using the XYZ pipetter to transfer DNA samples to separate test tubes carried by the plurality of test tube holders, to transfer at prescribed times a hybridization solution to the plurality of test tubes, to transfer at prescribed times a solution of magnetically responsive particles to the plurality of test tubes, and to selectively rotate at prescribed times the plurality of test tubes, such that binding reactions between the DNA samples, hybridization solution and magnetically responsive particles can occur and a precise, repeatable nucleic acid hybridization assay can be provided.

2. A process for performing a nucleic acid hybridization assay, comprising the steps of:

(a) providing a programmable XYZ pipetter having an aspirating/dispensing tip, and further providing an associated plurality of wells, an associated array of test tubes holders, each test tube holder being adapted to carry a test tube, means for effecting relative rotation between each of the test tubes and an associated magnetic field, about the test tube's longitudinal axis, and an associated microtiter plate;

(b) placing a hybridization solution in a first well, a solution of magnetically responsive particles in a second well, a wash buffer solution in a third well, a substrate buffer in a fourth well and a quench buffer solution in a fifth well;

(c) placing separate DNA samples in a plurality of test tubes carried in the array of test tube holders;

(d) using the XYZ pipetter to transfer a prescribed amount of the hybridization solution from the first well to each of the plurality of test tubes, in sequence, whereupon binding reactions are allowed to occur;

(e) using the XYZ pipetter to transfer a prescribed amount of the solution of magnetically responsive particles from the second well to each of the plurality of test tubes, in sequence;

(f) effecting relative rotation of the test tubes and their associate magnetic fields such that the magnetically responsive particles remain in liquid suspension and are allowed to undergo a binding reaction with the separate DNA samples;

(g) terminating the step of effecting relative rotation such that the magnetically responsive particles are moved by the magnetic fields to selected locations in the test tubes;

(h) using the XYZ pipetter to remove the unbound DNA sample and hybridization buffer from each of the plurality of test tubes, then to transfer the wash buffer solution from the third well to each of the

plurality of test tubes, and then to remove the wash buffer solution from each of a plurality of test tubes;

(i) using the XYZ pipetter to transfer the substrate buffer solution from the fourth well to each of the plurality of test tubes;

(j) effecting relative rotation of the test tubes and their associated magnetic fields such that the magnetically responsive particles remain in liquid suspension where an enzymatic process is allowed to occur;

(k) terminating the step of effecting relative rotation such that the magnetically responsive particles are moved by the magnetic fields to selected locations in the test tubes; and

(l) using the XYZ pipetter to transfer the quench buffer solution from the fifth well to each of the plurality of test tubes, and then to transfer a sample from each of the plurality of test tubes to a separate well of the microtiter plate, whereby the degree of binding reaction between the hybridization solution and the separate DNA samples can conveniently be assayed.

3. A process for performing a nucleic acid hybridization assay as defined in claim 2, wherein:

the XYZ pipetter is further associated with an optical sensor; and

the steps (f) and (j) of effecting relative rotation and the steps (g) and (k) of terminating are accomplished by moving the sampling tip of the XYZ

pipetter to a selected location where it can be detected by the optical sensor.

4. A process for performing a nucleic acid hybridization assay as defined in claim 2, wherein:

the step (e) of using the XYZ pipetter occurs a prescribed first time duration after the step (d) of using the XYZ pipetter, the first time duration being prescribed to ensure that any binding reactions between the hybridization buffer and the DNA samples are substantially completed;

the step (h) of using the XYZ pipetter occurs a prescribed second time duration after the step (e) of using the XYZ pipetter, the second time duration being prescribed to ensure that any binding reactions between the microscopic particles and the DNA samples are substantially completed; and

the step (l) of using the XYZ pipetter occurs a prescribed third time duration after the step (i), the third time duration being prescribed to allow an enzymatic process to occur.

5. The process for performing a nucleic acid hybridization assay as defined in claim 2, wherein in the step (h) of using the XYZ pipetter and the substeps of transferring the wash buffer and removing the wash buffer are repeated, sequentially, a plurality of times.

6. The process for performing a nucleic acid hybridization assay as defined in claim 2, wherein:

the XYZ pipetter is further associated with a wash station for washing the sampling tip; and

the process further includes steps of using the wash station to wash the sampling tip immediately following each use of the tip to transfer a solution from one of the plurality of wells to one of the plurality of test tubes or to remove a solution from one of the plurality of test tubes.

7. The process for performing a nucleic acid hybridization assay as defined in claim 2, wherein:

the hybridization solution includes DNA probes having an attached enzyme and DNA probes having attached biotin molecules; and

attached to the magnetically responsive particles are avidin molecules.

8. The process for performing a nucleic acid hybridization assay as defined in claim 7, wherein:

the enzyme exhibits fluorescence; and

the process further includes a step of assaying the samples carried in the microtiter plate using a fluorometer.

9. The process for performing a nucleic acid hybridization assay as defined in claim 2, further comprising performing an amplification step on the sample in the thermal cycler prior to step c.

10. The process of claim 9, wherein said amplification step is repeated at least once.

11. The process for performing a nucleic acid hybridization assay as defined in claim 2, further comprising performing a sample preparation step using affinity capture prior to step c.

12. In a process for performing a nucleic acid hybridization assay, the improvement comprising utilizing magnetically responsive particles to separate single stranded from double stranded nucleic acid.

13. In the process of claim 12, wherein the improvement further comprises utilizing magnetically responsive particles having avidin stably attached thereto through a covalent spacer.

14. A composition of matter comprising magnetically responsive particles having a ligand attached thereto through a covalent linker comprising multiple alternate hydrocarbon and amide residues.

15. The composition of matter of claim 14, wherein said linker comprises at least about three total residues.

16. The composition of matter of claim 14, wherein said ligand is avidin or streptavidin.

17. The composition of matter of claim 14, wherein said ligand is an antibody.

18. A composition of matter comprising magnetically responsive particles derivatized with more than about 100 pmoles of ligand binding sites per mg of dry particles.

19. The composition of matter of claim 18, wherein said ligand is avidin or streptavidin.

20. The composition of matter of claim 18, wherein said ligand is an antibody.

21. A method of producing magnetically responsive particles useful in a nucleic acid hybridization assay, comprising the steps of:

derivatizing said magnetically responsive particles with an amine or carboxyl terminal group; and successively adding moieties containing terminal amine or carboxyl groups so as to form multiple alternating hydrocarbon and amide residues.

FIG. 1

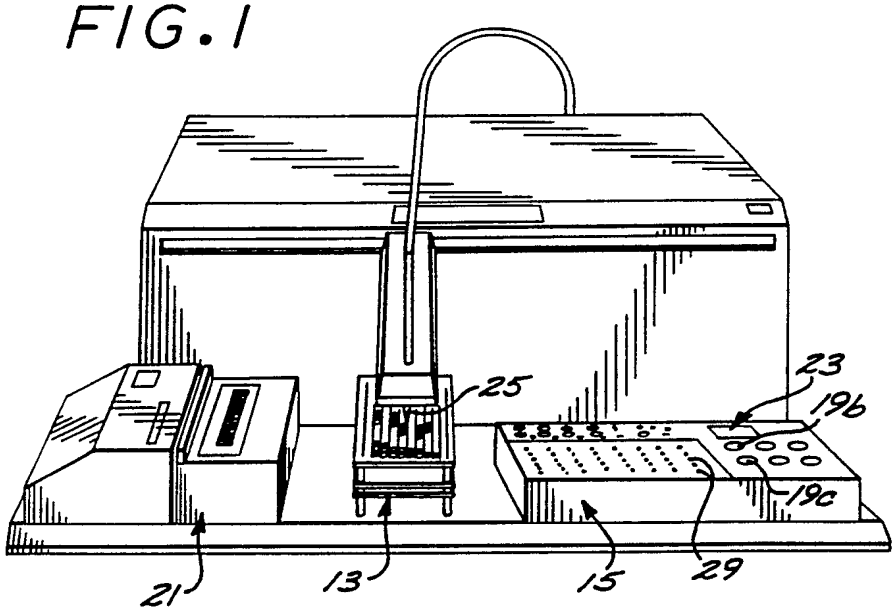
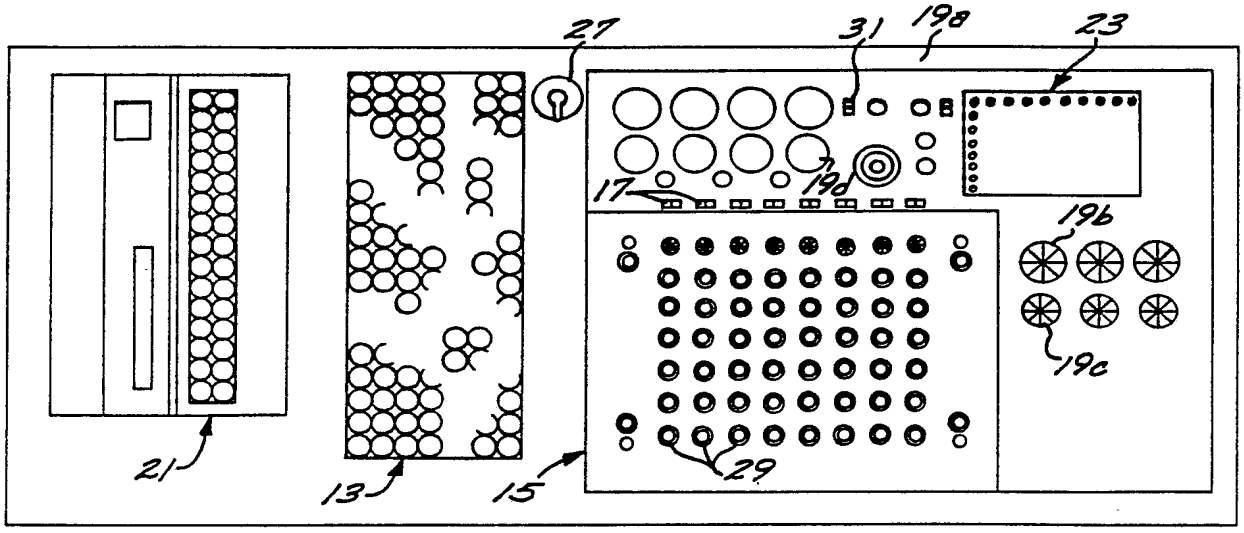


FIG. 2



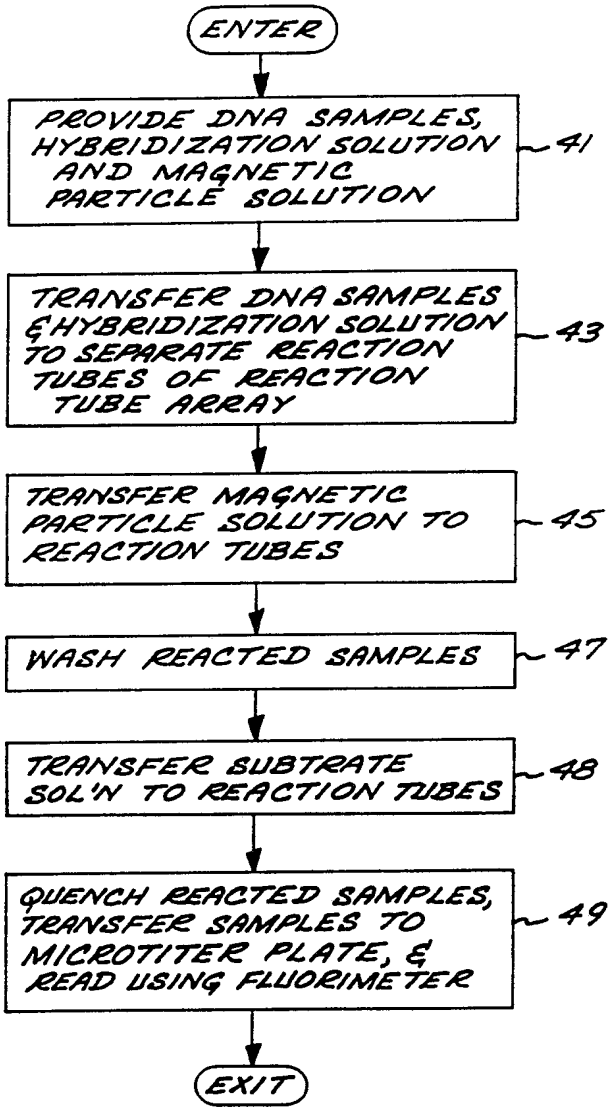

ENTER
PROVIDE DNA SAMPLES, HYBRIDIZATION SOLUTION AND MAGNETIC PARTICLE SOLUTION
41
TRANSFER DNA SAMPLES & HYBRIDIZATION SOLUTION TO SEPARATE REACTION TUBES OF REACTION TUBE ARRAY
43
TRANSFER MAGNETIC PARTICLE SOLUTION TO REACTION TUBES
45
WASH REACTED SAMPLES
47
TRANSFER SUBTRATE SOL'N TO REACTION TUBES
48
QUENCH REACTED SAMPLES, TRANSFER SAMPLES TO MICROTITER PLATE, & READ USING FLUORIMETER
49
EXIT

FIG. 3

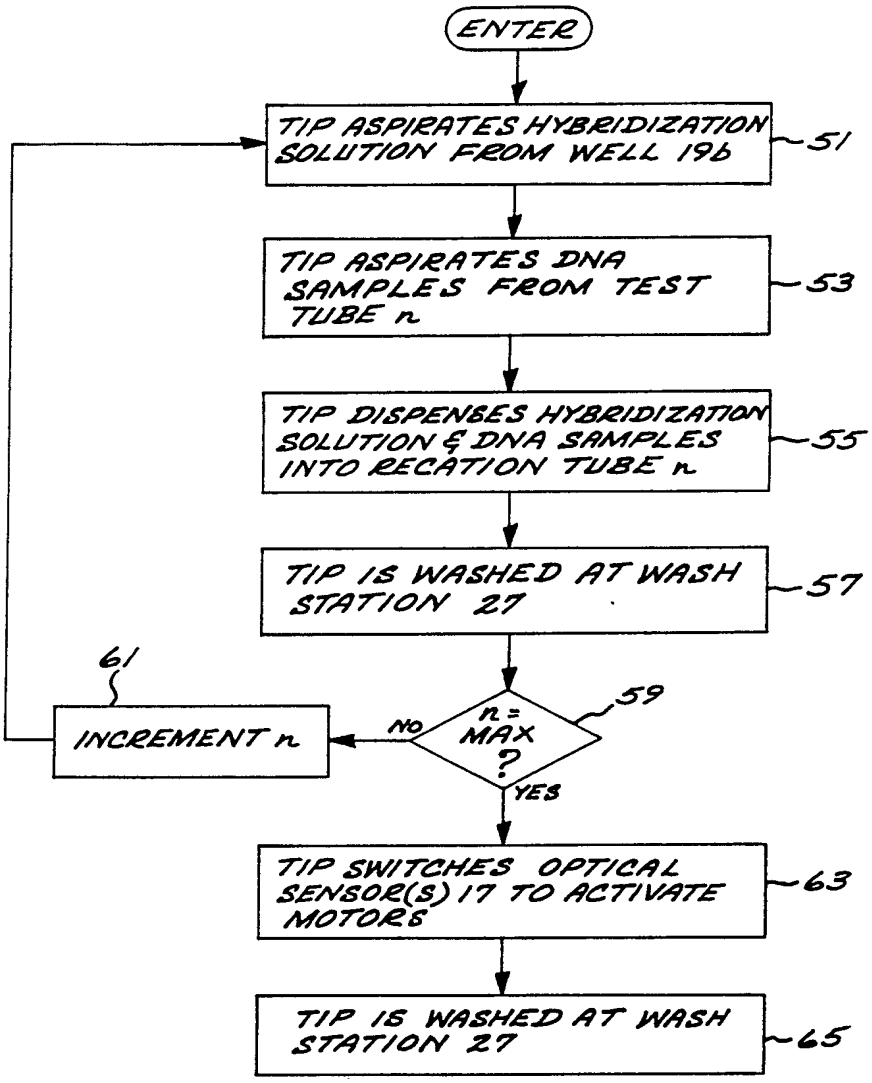

ENTER
TIP ASPIRATES HYBRIDIZATION SOLUTION FROM WELL 19b
51
TIP ASPIRATES DNA SAMPLES FROM TEST TUBE n
53
TIP DISPENSES HYBRIDIZATION SOLUTION & DNA SAMPLES INTO REACTION TUBE n
55
TIP IS WASHED AT WASH STATION 27
57
61
INCREMENT n
NO
n = MAX ?
59
YES
TIP SWITCHES OPTICAL SENSOR(S) 17 TO ACTIVATE MOTORS
63
TIP IS WASHED AT WASH STATION 27
65

FIG. 4

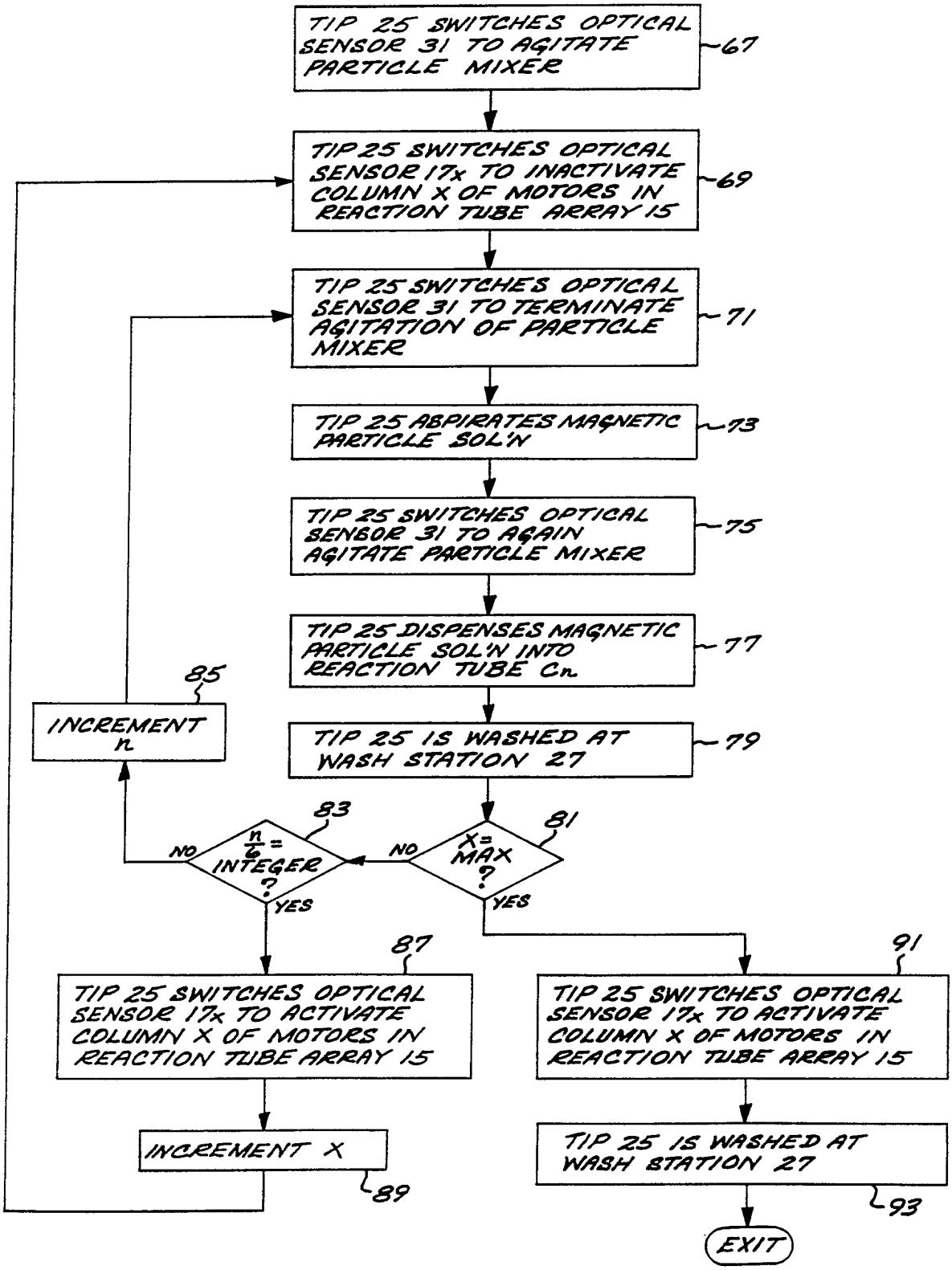
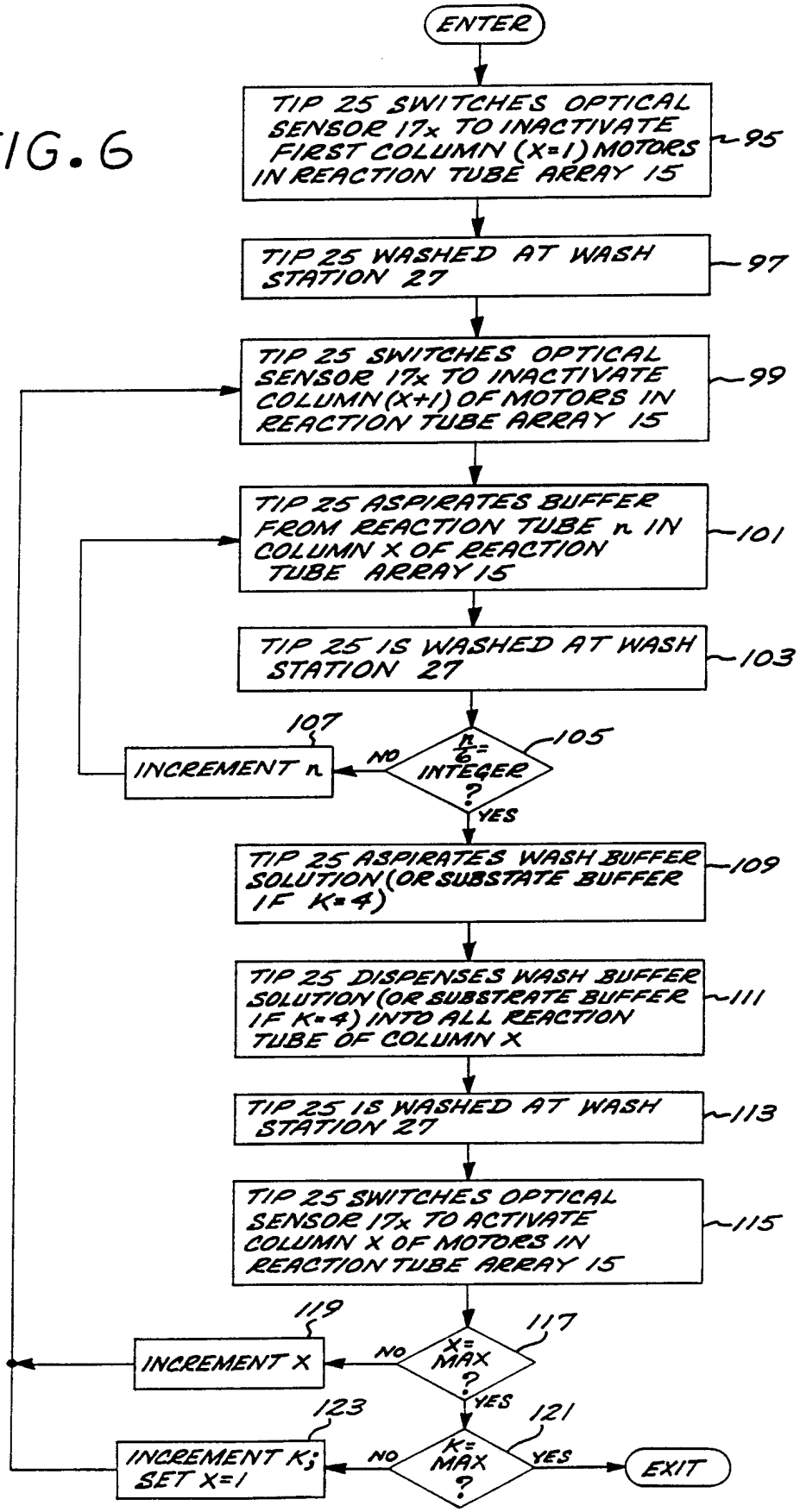
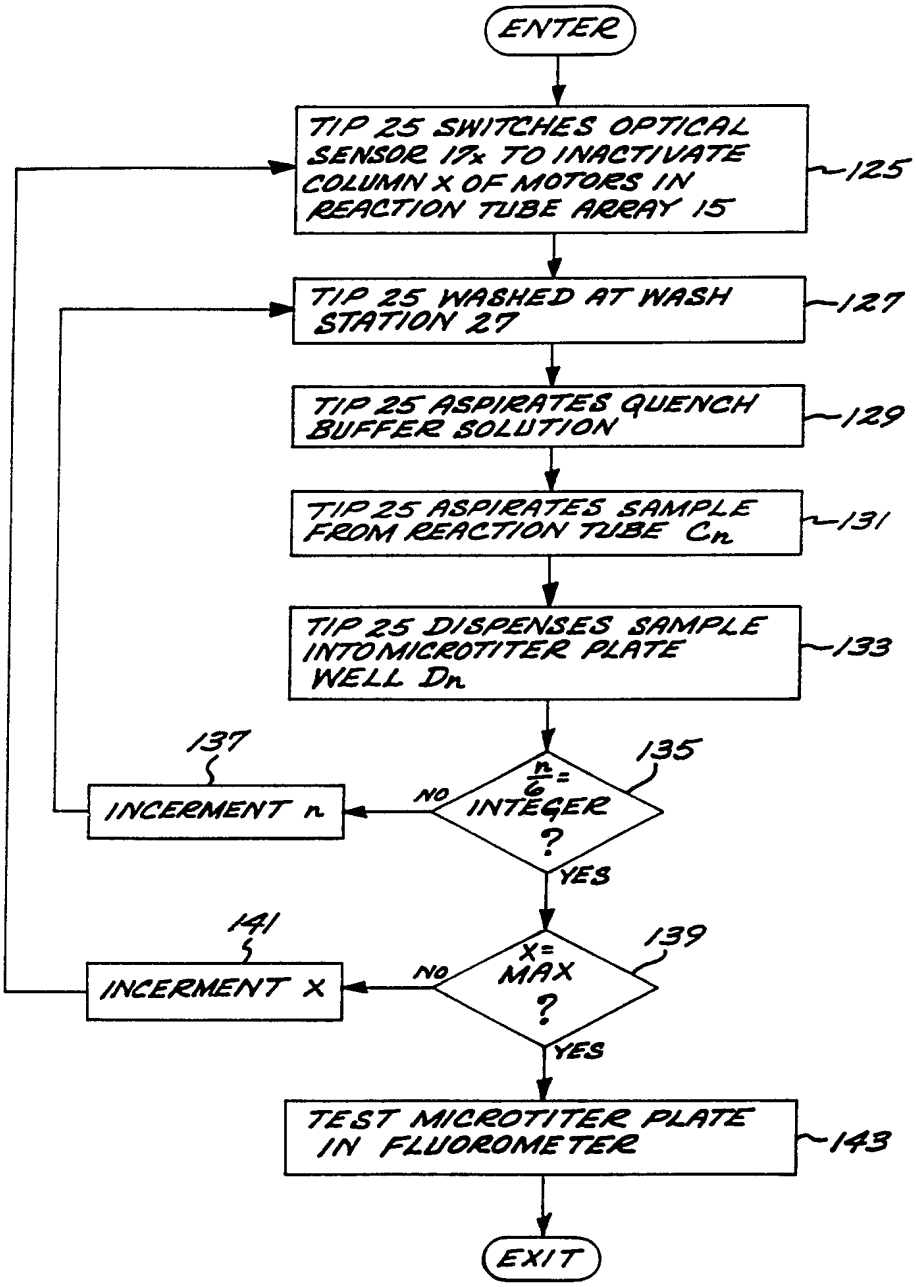

TIP 25 SWITCHES OPTICAL SENSOR 31 TO AGITATE PARTICLE MIXER
67
TIP 25 SWITCHES OPTICAL SENSOR 17x TO INACTIVATE COLUMN X OF MOTORS IN REACTION TUBE ARRAY 15
69
TIP 25 SWITCHES OPTICAL SENSOR 31 TO TERMINATE AGITATION OF PARTICLE MIXER
71
TIP 25 ABPIRATES MAGNETIC PARTICLE SOL'N
73
TIP 25 SWITCHES OPTICAL SENSOR 31 TO AGAIN AGITATE PARTICLE MIXER
75
TIP 25 DISPENSES MAGNETIC PARTICLE SOL'N INTO REACTION TUBE Cn
77
TIP 25 IS WASHED AT WASH STATION 27
79
X= MAX ?
81
NO
YES
n/6 = INTEGER ?
83
NO
YES
INCREMENT n
85
TIP 25 SWITCHES OPTICAL SENSOR 17x TO ACTIVATE COLUMN X OF MOTORS IN REACTION TUBE ARRAY 15
87
INCREMENT X
89
TIP 25 SWITCHES OPTICAL SENSOR 17x TO ACTIVATE COLUMN X OF MOTORS IN REACTION TUBE ARRAY 15
91
TIP 25 IS WASHED AT WASH STATION 27
93
EXIT

FIG. 5

FIG. 6


ENTER
TIP 25 SWITCHES OPTICAL SENSOR 17x TO INACTIVATE FIRST COLUMN (X=1) MOTORS IN REACTION TUBE ARRAY 15
95
TIP 25 WASHED AT WASH STATION 27
97
TIP 25 SWITCHES OPTICAL SENSOR 17x TO INACTIVATE COLUMN (X+1) OF MOTORS IN REACTION TUBE ARRAY 15
99
TIP 25 ASPIRATES BUFFER FROM REACTION TUBE n IN COLUMN X OF REACTION TUBE ARRAY 15
101
TIP 25 IS WASHED AT WASH STATION 27
103
n/6 = INTEGER ?
105
NO
YES
INCREMENT n
107
TIP 25 ASPIRATES WASH BUFFER SOLUTION (OR SUBSTATE BUFFER IF K=4)
109
TIP 25 DISPENSES WASH BUFFER SOLUTION (OR SUBSTRATE BUFFER IF K=4) INTO ALL REACTION TUBE OF COLUMN X
111
TIP 25 IS WASHED AT WASH STATION 27
113
TIP 25 SWITCHES OPTICAL SENSOR 17x TO ACTIVATE COLUMN X OF MOTORS IN REACTION TUBE ARRAY 15
115
X = MAX ?
117
NO
YES
INCREMENT X
119
K = MAX ?
121
NO
YES
EXIT
INCREMENT K; SET X=1
123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TIP 25 SWITCHES OPTICAL SENSOR 17x TO INACTIVATE COLUMN X OF MOTORS IN REACTION TUBE ARRAY 15
125
TIP 25 WASHED AT WASH STATION 27
127
TIP 25 ASPIRATES QUENCH BUFFER SOLUTION
129
TIP 25 ASPIRATES SAMPLE FROM REACTION TUBE Cn
131
TIP 25 DISPENSES SAMPLE INTO MICROTITER PLATE WELL Dn
133
n/6 = INTEGER ?
135
NO
INCERMENT n
137
YES
X= MAX ?
139
NO
INCERMENT X
141
YES
TEST MICROTITER PLATE IN FLUOROMETER
143
EXIT

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/02323

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: G 01 N 33/543, 35/00, 35/06, C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	WO, A, 8806632 (AVANCED MAGNETICS INC.) 7 September 1988 see page 8, line 15 - page 10, line 10; page 23, line 11 - page 24, line 30; page 30, line 20 - page 31, line 30; claims 1-7	12
A	--	14,21
A	WO, A, 8904373 (BAXTER INTERNATIONAL INC.) 18 May 1989 see page 2, lines 1-25; page 9, line 7 - page 10, line 18; page 25, lines 15-32	17,18
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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28th June 1991	02. 09. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Danielle van der Haas

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	pages 6157-6158 see the whole article ----------	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9102323 SA 46590

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 27/08/91
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